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Patent Office
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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 1321 for a patent by TASMANIAN ALKALOIDS PTY LTD filed on 14 January 1998.

WITNESS my hand this Sixteenth
day of February 1999

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MANAGER EXAMINATION SUPPORT AND SALES

AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION

FOR THE INVENTION ENTITLED:-

"IMPROVED PRODUCTION OF RETICULINE"

The invention is described in the following statement:-

IMPROVED PRODUCTION OF RETICULINE

The present invention relates to the improved production of reticuline. More particularly, the present invention relates to the use of a mutagenized *Papaver somniferum* poppy plant to produce (S)-reticuline in higher yield.

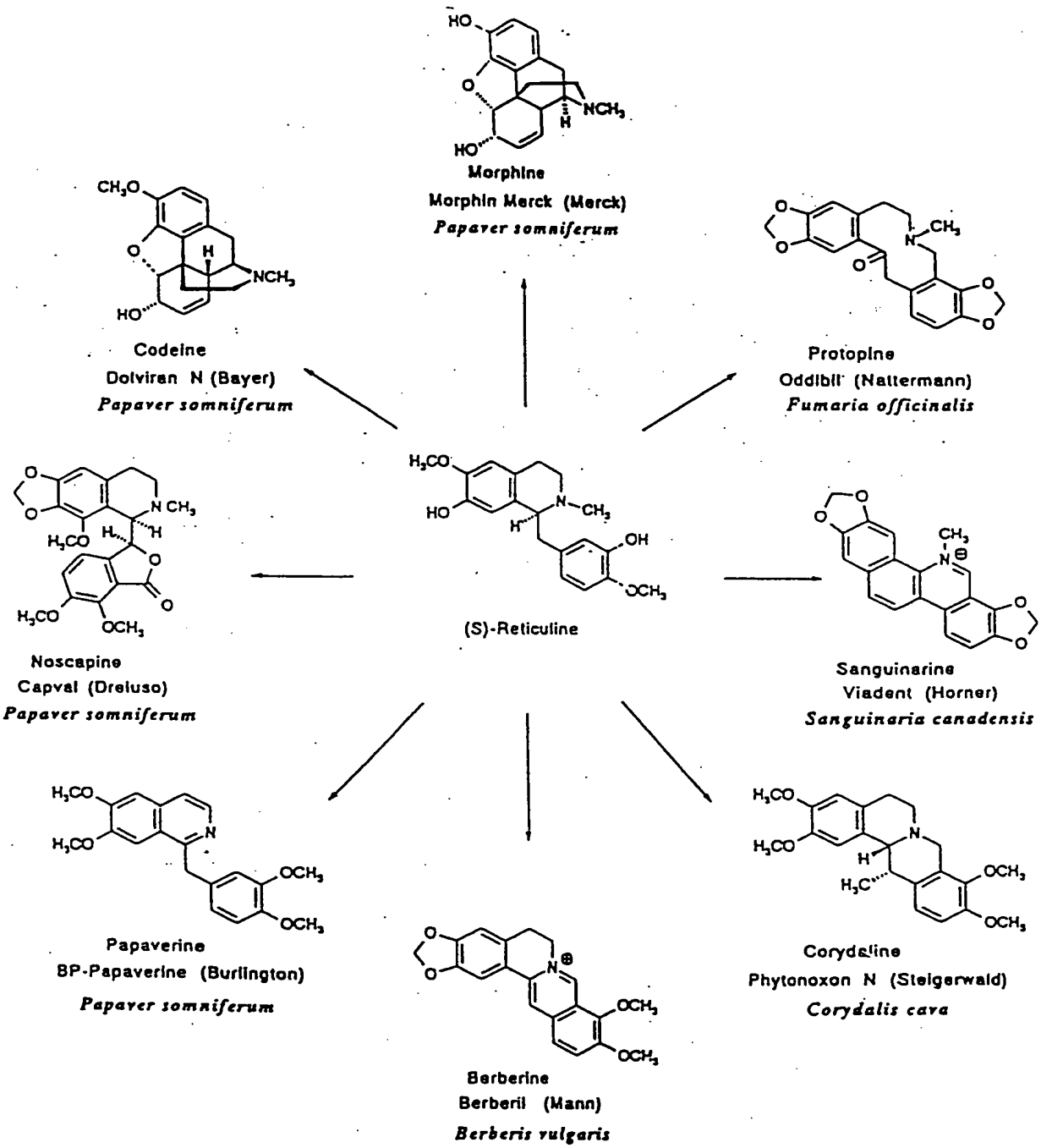
BACKGROUND OF THE INVENTION

(S)-Reticuline is an intermediate in the biosynthetic pathway leading to phenanthrene alkaloids such as codeine and morphine, phthalidisoquinoline alkaloids such as noscapine and benzylisoquinoline alkaloids such as papaverine in the *Papaver somniferum* poppy. (Scheme 1, from a paper to be published by Dr T.M. Kutchan). (S)-Reticuline is present in other plants, such as *Escholzia californica*, *Corydalis cava*, *Fumaria officinalis*, *Berberis vulgaris* and *Sanguinaria canadensis*, and has been identified as a precursor of protopine, benzo[c]phenanthridine alkaloids such as sanguinarine, protoberberine alkaloids such as corydaline and berberine itself.

These compounds are pharmaceutically useful, for example, the analgesic properties and commercial value of codeine and morphine require little introduction. Noscapine is a useful antitussive compound. Papaverine is a smooth muscle relaxant and a cerebral vasodilator. Berberine has been used as an antibacterial, antimalarial and antipyretic compound.

As well as being an important precursor for numerous pharmaceutical products, (S)-reticuline has recently been shown to accelerate hair growth in cultured hair cells (Biol. Pharm. Bull., 20(5) 586-588 (1997)).

SCHEME 1


Morphine
Morphin Merck (Merck)
Papaver somniferum
Codeine
Dolviran N (Bayer)
Papaver somniferum
Protopine
Oddibil (Nattermann)
Fumaria officinalis
(S)-Reticuline
Noscapine
Capval (Dreluso)
Papaver somniferum
Sanguinarine
Viadent (Horner)
Sanguinaria canadensis
Papaverine
BP-Papaverine (Burlington)
Papaver somniferum
Berberine
Berberil (Mann)
Berberis vulgaris
Corydaline
Phytonoxon N (Steigerwald)
Corydalis cava

(±)-Reticuline has been synthesised, by a lengthy and difficult synthesis (Tomita, M. and Kikkawa, I., Pharm Bull Japan, 4, 230 (1956), Chem Abs, 51, 8116 (1957) and Gopinath K .W., Govindachari, T.R., and Viswanathan N, Ber, 92, 1657 (1959)).

The synthesis of the (S) form has also been reported by Konda et al, Chem Pharm Bull, 23, 1063 (1975). Whilst effective, the difficulty of the totally synthetic route is that only small quantities of the compound are available after a long and costly synthesis. Thus, total synthesis is undesirable as a means of making substantial quantities of (S)-reticuline.

A second reason for the limited availability and high cost of (S)-reticuline is that it is present in source plants at very low concentrations. For instance it is found in commercial poppy straw at 0.04%, and it is present in the opium of *Papaver somniferum* in trace amounts (Brochman-Hanssen, E. and Furaya, T., Planta Med. 12, 328 (1964)). Due to the low concentrations of (S)-reticuline in the various plant sources, there is at present no commercial source of (S)-reticuline.

(S)-Reticuline has been isolated from opium by conventional but lengthy extraction procedures. The initial step involves the mixing of powdered opium with a cationic exchange resin in hot water. The alkaloids adsorb to the ion exchange resin and the non polar fractions which are not of interest are removed by washing. The alkaloid fractions are removed by elution with methanol and can be extracted into organic solvents, such as chloroform, by using controlled acid/base extractions: for example, see the work by Brochmann-Hanssen and Furuya, 1964, Planta Med. 12, 328 and references cited therein.

Such an extraction process is expensive and involve considerable losses of opium derived material. The yield of (S)-reticuline from opium is low, Brochmann-Hanssen

and Furuya reporting that it represents about 0.15% of the total opium mass. These factors all combine to render (S)-reticuline extraction from opium commercially unattractive.

Alkaloids are extracted from the poppy capsules of *Papaver somniferum* by two commercial methods. In one method, the immature capsule is cut and the latex collected from the wound and air dried to produce opium. In a second method, the mature poppy capsules and the poppy capsule stems are collected, and threshed to remove the seeds and form a straw. When necessary, the straw is dried to a water content below 16%. Solvent or water extraction is employed to remove the alkaloids from the straw.

Where solvent, water or super critical fluid, such as CO_2, extraction is employed to remove the alkaloids from the straw, such method, as practised, involves the production of "Concentrate of Poppy Straw". Concentrate of Poppy Straw has been defined as "The material arising when poppy straw has entered into a process for the concentration of its alkaloids, when such material is made available in trade," (Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances Under International Control, United Nations, New York, 1983). For the purposes of the present specification, Concentrate of Poppy Straw is taken to mean "the crude extract of poppy straw in either liquid, solid or powder form". When in liquid form, the liquid is preferably concentrated before entering into commerce. The generally preferred Concentrate of Poppy Straw is the powder form which results from simply removing the solvent or water following extraction of the poppy straw.

As the synthesis of (S)-reticuline is economically impractical, and extraction from natural sources is low yielding and requires extensive purification, it would be

desirable to increase production by increasing the amount of (S)-reticuline produced by a plant.

It is also desirable to increase the ratio of (S)-reticuline to phenanthrene-type alkaloids in the plant and the plant products. Phenanthrene alkaloids are those incorporating the phenanthrene ring system into their structure. Morphine, codeine, thebaine and oripavine are examples of such a phenanthrene type alkaloid. Reticuline however does not include this structural element but instead is based on benzyl-isoquinoline as its major structural element.

Surprisingly, the present inventors have found a method of increasing (S)-reticuline production and the (S)-reticuline to phenanthrene alkaloid ratio by modifying *Papaver somniferum*.

It is an object of the present invention to provide a commercially viable alternative to the methods in the prior art.

It will be understood by a skilled addressee that the present invention, whilst exemplified in relation to *Papaver somniferum*, would be equally applicable to other plants in which (S)-reticuline is present, such as *Escholzia californica*, *Corydalis cava*, *Fumaria officinalis*, *Berberis vulgaris* and *Sanguinaria canadensis*.

In the context of the present invention, the term "opium" is taken to include material which is obtained from a modified *Papaver somniferum* in a similar fashion to that used to obtain opium (as conventionally defined) from a non-modified plant.

SUMMARY OF THE INVENTION

In a broad aspect, the invention consists in a stably reproducing *Papaver somniferum* having an (S)-reticuline content higher than that of a native *Papaver somniferum*.

In another aspect, the invention consists in a stably reproducing *Papaver somniferum*, which upon the harvesting of the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw obtained from a native *Papaver somniferum*.

In another aspect, the invention consists in a stably reproducing *Papaver somniferum*, which upon the collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)-reticuline content higher than the latex obtained from a native *Papaver somniferum*.

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of (S)-reticuline oxidase is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having a (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of (S)-reticuline oxidase is inhibited, with the result that upon collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)- reticuline content higher than that of native *Papaver somniferum*.

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of dehydroreticuline reductase is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*,

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of dehydroreticuline reductase is

inhibited, with the result that upon the collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)-reticuline content higher than the opium of a native *Papaver somniferum*.

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*,

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon the collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)-reticuline content higher than the opium of a native *Papaver somniferum*.

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: (S)-reticuline oxidase, dehydroreticuline reductase or berberine bridge enzyme (BBE) are inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*

In another aspect, the invention consists in a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: (S)-reticuline oxidase, dehydroreticuline reductase or berberine bridge enzyme (BBE) are inhibited, with the result that upon collection and drying of the

latex from the immature poppy capsules will yield an opium having an (S)-reticuline content higher than that of native *Papaver somniferum*.

Preferably, such stably reproducing *Papaver somniferum* yield a poppy straw having an (S)-reticuline content greater than 1.0%, and more preferably greater than 2.5%.

Preferably, such stably reproducing *Papaver somniferum* yield an opium having an (S)-reticuline content greater than 10%, and more preferably greater than 20%.

According to another aspect, the invention consists in a seed yielding a stably reproducing *Papaver somniferum* according to any one of the preceding aspects.

According to another aspect, the invention consists in an improved poppy straw of a stably reproducing *Papaver somniferum*, the threshed straw having an (S)-reticuline content higher than that of the poppy straw of a native *Papaver somniferum*.

Preferably, such stably reproducing *Papaver somniferum* yield a poppy straw having an (S)-reticuline content greater than 1.0%, and more preferably greater than 2.0%.

According to another aspect, the invention consists in an improved opium of a stably reproducing *Papaver somniferum*, the opium having an (S)-reticuline content higher than that of the opium of a native *Papaver somniferum*. Preferably, such stably reproducing *Papaver somniferum* yield an opium having an (S)-reticuline content greater than 10%, and more preferably greater than 20%.

According to another aspect the invention consists in an improved concentrate of poppy straw of a stably reproducing *Papaver somniferum*, the concentrate of poppy straw having an (S)-reticuline content higher than that of the concentrate of poppy straw of a native *Papaver somniferum*.

Preferably, such stably reproducing *Papaver somniferum* yields a concentrate of poppy straw having an (S)-reticuline content greater than 30%, and more preferably greater than 60%.

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum*, which upon the harvesting of their poppy capsules will yield a poppy straw having an (S)-reticuline content higher than that of the poppy straw of a native *Papaver somniferum*.

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum*, which upon the collection and drying of the latex from their immature poppy capsules will yield an opium having an (S)-reticuline content higher than that of the opium of a native *Papaver somniferum*.

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of (S)-reticuline oxidase is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*

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According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of dehydroreticuline reductase is inhibited, with the result that upon harvesting the poppy

capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum.*

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of dehydroreticuline reductase is inhibited, with the result that upon the collection and drying of the latex from their immature poppy capsules will yield an opium having an (S)-reticuline content higher than the opium content of native *Papaver somniferum.*

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon the collection and drying of the latex from their immature poppy capsules will yield an opium having an (S)-reticuline content higher than the opium content of native *Papaver somniferum.*

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: dehydroreticuline reductase, (S)-reticuline oxidase or berberine bridge enzyme (BBE) are inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline content higher than the poppy straw of a native *Papaver somniferum*

According to another aspect the invention consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: dehydroreticuline reductase, (S)-reticuline oxidase or berberine bridge enzyme (BBE) are inhibited, with the result that upon the collection and drying of the latex from their immature poppy capsules will yield an opium having an (S)-reticuline content higher than the opium content of native *Papaver somniferum*.

Preferably, such stably reproducing *Papaver somniferum* stands yield a poppy straw having an (S)-reticuline content greater than 1.0%, and more preferably greater than 2.0%.

Preferably, such stably reproducing *Papaver somniferum* stands yield an opium having an (S)-reticuline content greater than 10%, and more preferably greater than 20%

According to another aspect the invention consists in a method for the production of (S)-reticuline which comprises the steps of:

a) harvesting poppy capsules of a stably reproducing *Papaver somniferum* to produce a straw where the plant is such a plant that the straw has a higher (S)-reticuline content than the straw of a native *Papaver somniferum*, and

b) chemically extracting the (S)-reticuline from the straw.

According to another aspect the invention consists in a method for the production of (S)-reticuline which comprises the steps of:

a) collecting and drying the latex of the immature poppy capsules of a stably reproducing *Papaver somniferum* to produce opium where the plant is such a plant that the opium has a (S)-reticuline content higher than that of the opium of a native *Papaver somniferum*, and

b) chemically extracting the (S)-reticuline from the opium.

Preferably, in such methods, stably reproducing *Papaver somniferum* yield a poppy straw having an (S)-reticuline content greater than 1.0%, and more preferably greater than 2.0%.

Preferably, in such methods stably reproducing *Papaver somniferum* yield an opium having an (S)-reticuline content greater than 10%, and more preferably greater than 20%.

The invention also consists in (S)-reticuline when obtained by any of the forgoing processes.

According to another aspect the invention consists in a method to improve the (S)-reticuline yield of a stably reproducing *Papaver somniferum*, the method comprising the steps of:

a) exposing at least one poppy seed of *Papaver somniferum* to a mutagenizing agent,

b) growing the at least one poppy seed to produce a plant bearing a leaf or an immature poppy capsule, optionally through multiple self fertilized generations,

c) sampling the leaf or poppy capsule for the presence of (S)-reticuline, morphine and codeine, and

d) repeating steps a) to c) until a poppy plant of *Papaver somniferum* is obtained having a (S)-reticuline content higher than that of a native *Papaver somniferum*.

Preferably steps a) to c) are repeated until the (S)-reticuline content shows no further increase on mutagenesis.

The invention also consists in an improved poppy straw of a stably reproducing *Papaver somniferum*, the threshed straw having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in an improved opium of a stably reproducing *Papaver somniferum*, the opium having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in an improved concentrate of poppy straw of a stably reproducing *Papaver somniferum*, the concentrate of poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stably reproducing *Papaver somniferum*, which upon the harvesting of the poppy capsules will yield a poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater.

The invention also consists in a stably reproducing *Papaver somniferum*, which upon the collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stably reproducing *Papaver somniferum* in which the production or activity of (S)-reticuline oxidase is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stably reproducing *Papaver somniferum* in which the production or activity of (S)-reticuline oxidase is inhibited, with the result that upon the collection and drying of the latex from the immature poppy capsules will yield an

opium having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

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The invention also consists in a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon the collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: (S)-reticuline oxidase, dehydroreticuline reductase or berberine bridge enzyme (BBE) are inhibited, with the result that upon harvesting the poppy capsules will yield a poppy

straw having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: (S)-reticuline oxidase, dehydroreticuline reductase or berberine bridge enzyme (BBE) are inhibited, with the result that upon the collection and drying of the latex from the immature poppy capsules will yield an opium having an (S)-reticuline to phenanthrene alkaloid ratio of about 100% or greater by weight.

The invention also consists in a stand of a stably reproducing *Papaver somniferum*, which upon the harvesting of their poppy capsules will yield a poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight.

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The invention also consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon harvesting the poppy capsules will yield a poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight.

The invention also consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of berberine bridge enzyme (BBE) is inhibited, with the result that upon the collection and drying of the latex from their immature poppy capsules will yield an opium having an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight.

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capsules will yield a poppy straw having an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight.

The invention also consists in a stand of a stably reproducing *Papaver somniferum* in which the production or activity of two or more enzymes selected from the group comprising: (S)-reticuline oxidase, dehydroreticuline reductase or berberine bridge enzyme (BBE) are inhibited, with the result that upon the collection and drying of the latex from their immature poppy capsules will yield an opium having an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight.

The invention also consists in a method for the production of (S)-reticuline which comprises the steps of:

a) harvesting poppy capsules of a stably reproducing *Papaver somniferum* to produce a straw where the plant is such a plant that the straw has an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight, and

b) chemically extracting the (S)-reticuline from the straw.

The invention also consists in a method for the production of (S)-reticuline which comprises the steps of:

a) collecting and drying the latex of the immature poppy capsules of a stably reproducing *Papaver somniferum* to produce opium where the plant is such a plant that has an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight, and

b) chemically extracting the (S)-reticuline from the opium.

The invention also consists in a method to improve the (S)-reticuline yield of a stably reproducing *Papaver somniferum*, the method comprising the steps of:

a) exposing at least one poppy seed of *Papaver somniferum* to a mutagenizing agent,

b) growing the at least one poppy seed to produce a plant bearing a leaf or an immature poppy capsule, optionally through multiple self fertilized generations,

c) sampling the leaf or poppy capsule for the presence of (S)-reticuline, morphine and codeine, and

d) repeating steps a) to c) until a poppy plant of *Papaver somniferum* is obtained having an (S)-reticuline to phenanthrene alkaloid ratio of 100% or greater by weight.

Preferably in the aforementioned products and methods, the (S)-reticuline to phenanthrene alkaloid ratio is 200% or greater by weight.

More preferably the (S)-reticuline to phenanthrene alkaloid ratio is 1250% or greater by weight.

It is also highly preferred that there are substantially no phenanthrene alkaloids present.

The invention also consists in (S)-reticuline when obtained from any of the forgoing plants or plant products.

Those skilled in the art will appreciate also that there are other methods of affecting the targeted enzymes to increase the accumulation of (S)-reticuline, such as transfection and targeting of genes and/or m-RNA encoding the production of (S)-reticuline oxidase, dihydroreticuline reductase and berberine bridge enzyme (BBE).

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows a HPLC trace of an extract of modified *Papaver somniferum* (bottom line) and an extract spiked with a standard for alkaloid analysis.

DETAILED DESCRIPTION OF THE INVENTION

Utilizing the mutagenized plants of *Papaver somniferum* as described herein, persons skilled in the art easily know how to grow and reproduce such plants, collect the latex or the dried straw and purify the (S)-reticuline. As one enablement of the present invention, seeds to the mutagenized plants of *Papaver somniferum*, as described herein, have been deposited under the Budapest Treaty with the Australian Government Analytical Laboratories (AGAL), The New South Wales Regional Laboratory, 1 Suakin Street, Pymble, NSW, 2073, Australia, on XXXXXX, under Accession No. XXXXX, and will be made available upon the maturation of this application into a patent. The availability of these seeds is not to be construed as a license to practice this invention in contravention of rights granted under the authority of any government in accordance with its patent or breeder's rights laws.

Methods of seed mutagenesis as well as mutagens suitable for use in these methods, such as, ethyl methanesulfonate (EMS), are described in the Manual on Mutation Breeding, 2nd ed., I.A.E.A., Vienna 1977 or in Plant Breeding, Principles and Prospects, Chapman and Hall, London 1993. For X-ray mutagenized seeds, hydrated seeds might be treated with 20,000 rads, (30cm from the source for 45 minutes using a filter). X-ray mutagenesis is described and compared to EMS mutagenesis by Filippetti, A. et al., "Improvement of Seed Yield in Vici Baba L. By Using Experimental Mutagenesis II Comparison of Gamma-Radiation and Ethyl-MethaneSulphonate (EMS) in Production of Morphological Mutants", Euphytica 35 (1986) 49-59. DEB, diepoxybutane, mutagenized seeds might be obtained by soaking the seeds in water overnight, then soaking in 22mM DEB for 4 hours, followed by extensive washing. Further mutagens include ethyl-2-chloroethyl sulphide, 2-chloroethyl-dimethylamine,

ethylene oxide, ethyleneimine, dimethyl sulphonate, diethyl sulphonate, propane sulphone, beta-propiolactone, diazomethane, N-methyl-N-nitrosourethane, acridine orange and sodium azide. The preferred mutagen employed herein is EMS.

Mutagenesis utilizing EMS is well described in the literature. The Manual on Mutation Breeding, supra, reports a preferred EMS mutagenesis process for barley seeds as practiced by K. Mikaelson. In this preferred process, the seeds are prepared, pre-soaked, treated with the mutagen and post-washed.

In the preparation, uniform size seeds are selected and placed in mesh polyethylene bags, about 200 seeds. Subsequently, the seeds are kept in a dessicator over a 60% glycerol solution, which gives the seeds a moisture content of about 13%. In pre-soak, the seed bags are transferred to beakers with distilled or deionized water and soaked for 16 - 20 hours at a temperature of 20 - 22°C. The pre-soak period is important to the uptake or diffusion of mutagen. The pre-soak should be sufficient to promote diffusion of the mutagen into the seed and at the same time stimulate the embryo meristem tissue to start DNA synthesis. It is at this point that high mutation frequency can be achieved with minimal chromosome damage. To treat with the mutagen, the seed bags are transferred to beakers containing a solution of EMS in distilled or deionized water. For barley and wheat, the maximal mutation frequencies are obtained under treatment conditions where the EMS concentration is 0.05 - 0.1 M, the bath temperature is 30 - 35°C, and the exposure time of the seeds to the bath is 0.5 - 2 hours. Relatively weak treatments are preferred in mass screening to achieve maximal mutation with minimal physiological damage. Such treatments give better germinability and survival, less plant growth reduction and less sterility compared with stronger treatments. A thorough post-wash in water after the EMS treatment is essential. This post-wash can be

carried out in running tap water, preferably at not less than 15°C, for a period of not less than 4 hours. The EMS should be removed by the post-wash in order to prevent uncontrollable after-effects by the mutagen. After post-washing, the seeds should be planted as soon as possible. If the seeds cannot be planted soon after the mutagenesis process, they should be immediately dried back to a moisture content of about 13%. This can be accomplished by simply air drying the seeds at room temperature and a reasonably low relative humidity.

Persons skilled in the art will recognize that this preferred mutagenesis method for barley and wheat seeds can be easily modified for poppy seeds. In the case of poppy seeds, it has been found useful and convenient by the inventors hereof to dispense with dessication, to extend the time of pre-soak to up to 48 hours and to lower the bath temperature of mutagen treatment to 20°C. Other modifications will be apparent to skilled practitioners.

After the seeds have been exposed to the mutagen, the seeds are grown to maturity in controlled conditions and self-pollinated. The seeds from the mature plant are taken and at least one seed is planted to grow an M2 generation. The M2 generation is screened for alkaloid production. Of course, it is possible to screen the M1 generation, but there are several advantages to screening the M2 generation. Firstly, screening the M2 generation insures that the trait resulting from mutagenesis can be inherited.

Secondly, by growing the M2 generation, the basic hardiness of the plant is proven before screening. Thirdly, traits resulting from mutagenesis are generally inherited as recessive genes, and these will be homozygous in the M2 generation, i.e., they will not be masked by a dominant gene. The M2 plants can be grown to produce an immature capsule, but it is possible to save time and labor if the plant is screened at an

earlier stage of growth. It is recommended that the plants be screened at a point beginning at the 10 leaf stage, up to the "running-up" stage, where the plant reaches about 6 inches in height. The screening process itself is the most labor intensive. Thus, to improve return on labor, only plants that appear healthy should be screened.

In the screening process, the objective is to measure each plant for alkaloids such as morphine, codeine, oripavine, thebaine, noscapine, papaverine and any other alkaloids which might occur as a result of blockage to one or more metabolic pathways, such as (S)-reticuline. The trait of a high (S)-reticuline content relative to other alkaloids is highly desirable, and once established is highly heritable. This can be accomplished by extracting, for example, a dry leaf into a liquid buffer or by dissolving a latex sample into a buffer. The buffer solutions are placed in glass vials and loaded into 96-place carousels and fed mechanically through any of the high-throughput HPLCs available on the market.

Plants with unusual alkaloid contents are grown further and examined in more detail. According to procedure herein, a second sample is taken from about 1/20 plants to clarify the results of the initial screen.

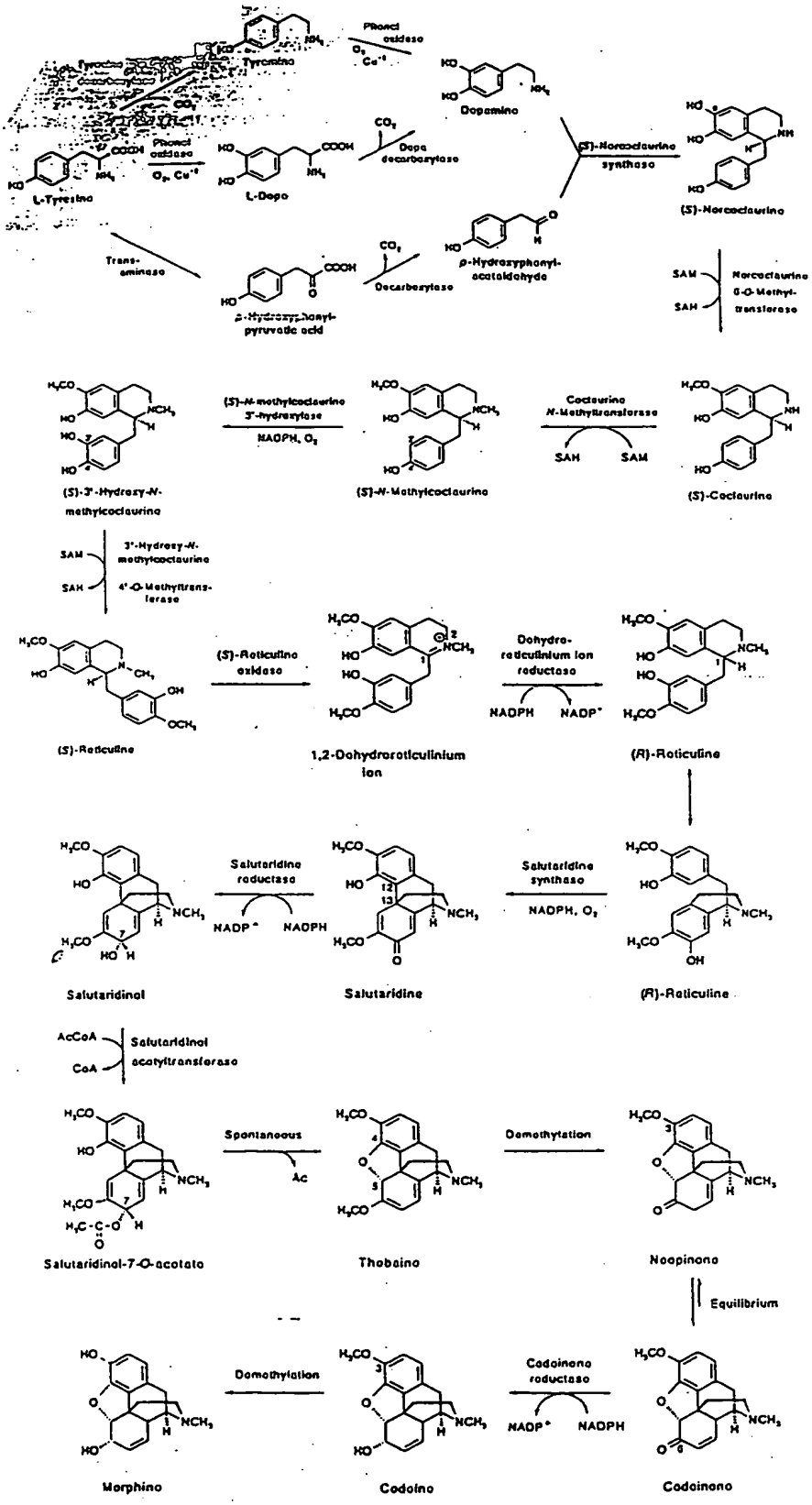
As stated above, there is obtained by the present invention, a threshed poppy straw or opium having an (S)-reticuline content higher than that observed in native plants. Preferably, there is substantially no codeine, morphine, thebaine or other phenanthrene alkaloid in the alkaloid combination.

The desired traits, i.e. high (S)-reticuline content versus phenanthrene alkaloid content, once established are highly heritable. To maintain the desired traits, care should be taken to prevent cross-pollination with normal plants unless such cross-pollination is part of a controlled breeding program.

The theory whereby mutagenesis has been found to be capable of raising the (S)-reticuline content of *Papaver somniferum* relative to the phenanthrene alkaloid content is not capable of a certain or definite explanation at this time. The mutagenesis may have resulted in the modification of certain enzyme activity in a qualitative or quantitative manner. Alternatively, the mutagenesis might have modified the biosynthesis pathway in any number of ways to minimize the production of morphine and codeine. Despite the fact that definite answers are not now available, there are good reasons to believe that the correct answer is known.

A postulated biosynthetic pathway in *Papaver somniferum* via (S)-reticuline to morphine is shown (Scheme 2, from a paper to be published by Dr T.M. Kutchan).

SCHEME 2


Tyrosine
Phenol oxidase
Dopamine
L-Tyrosine
L-Dopa
Dopa decarboxylase
(S)-Norcoclaurine synthase
(S)-Norcoclaurine
Trans-aminase
p-Hydroxyphenyl-acetaldehyde
Decarboxylase
SAM
SAH
Norcoclaurine 6-O-Methyl-transferase
(S)-3'-Hydroxy-N-methylcoclaurine
(S)-N-methylcoclaurine 3'-hydroxylase
NADPH, O2
(S)-N-Methylcoclaurine
Coclaurine N-Methyltransferase
(S)-Coclaurine
3'-Hydroxy-N-methylcoclaurine 4'-O-Methyltrans-ferase
(S)-Reticuline
(S)-Reticuline oxidase
1,2-Dehydroreticulinium ion
Dehydro-reticulinium ion reductase
NADPH
NADP+
(R)-Reticuline
Salutaridine reductase
Salutaridine synthase
Salutaridinol
Salutaridine
Salutaridinol acetyltransferase
AcCoA
CoA
Salutaridinol-7-O-acetate
Spontaneous
Ac
Thebaine
Demethylation
Neopinone
Equilibrium
Morphine
Codeine
Codeinone reductase
Codeinone

By the methods herein, a variety of *Papaver somniferum* was obtained having a high (S)-reticuline content and substantially no thebaine or morphine. Thus, it is believed, for the *Papaver somniferum* variety described herein, that the production or activity of (S)-reticuline oxidase has been substantially inhibited, resulting in a buildup of (S)-reticuline and less material following the biosynthetic pathway to its endpoint, i.e. morphine. It is also possible that the production or activity of dehydroreticuline reductase has been inhibited. By feedback inhibition through 1,2-dehydroreticuline, this would lead to an accumulation of (S)-reticuline.

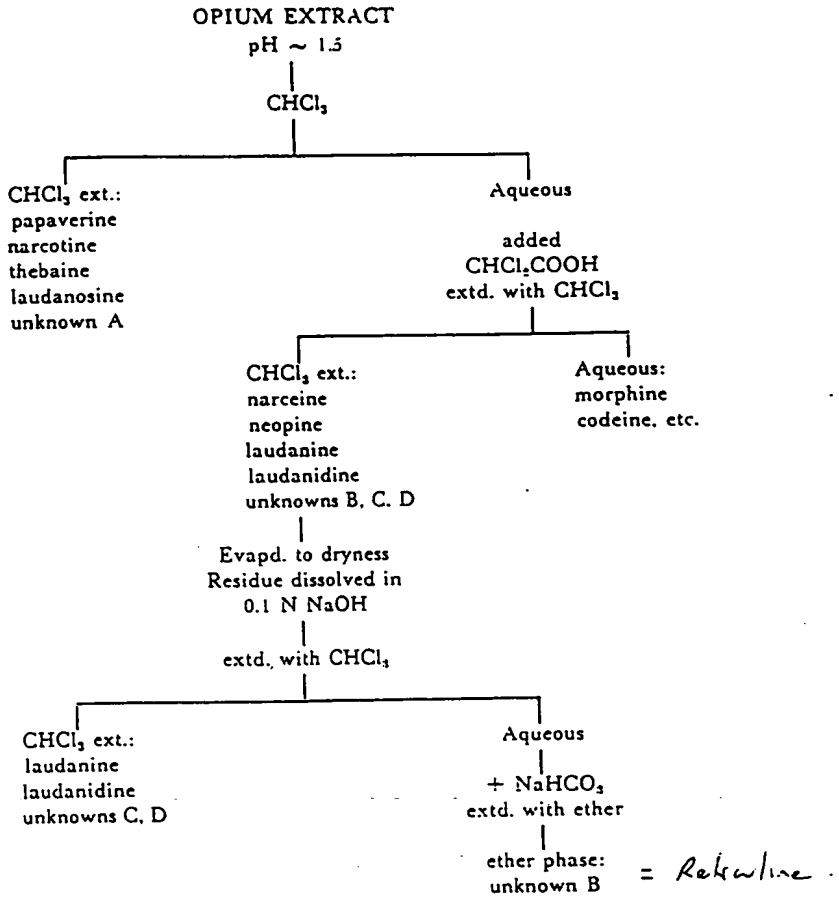
It is also possible that stably reproducing *Papaver somniferum* in accordance with the present invention may also be obtained by recombinant DNA techniques. In particular, after isolation and sequencing of the gene coding for (S)-reticuline oxidase, the gene or the mRNA transcript may be modified, deleted or blocked to inhibit or prevent the production of (S)-reticuline oxidase. Techniques for modifying or deleting specific regions of DNA sequences or blocking mRNA are well known to those skilled in the art.

It would also be possible to accumulate (S)-reticuline in other species by blocking particular enzymes. For example, in Berberis species, the berberine bridge enzyme could be blocked either using mutagenesis (as demonstrated here) or through recombinant DNA techniques.

Recovering (S)-reticuline from either the dried straw or from the opium of *Papaver somniferum* is a process well established in the art. A schematic diagram (Scheme 3) is shown outlining the process of (S)-reticuline extraction from the alkaloid containing extract of opium. This procedure was outlined by Brochmann-Hanssen and Furuya (Planta Med. 12, 328-333). Methods of obtaining of a highly acidic (pH 1.5)

opium extract were well known in the art and represented a common starting point for most alkaloid extractions from *Papaver somniferum*. Those skilled in the art will appreciate that presently there are a number of suitable starting materials for such extractions depending on the industrial process being used, and that Scheme 3 provides one example only.

SCHEME 3


OPIUM EXTRACT
pH ~ 1.5
CHCl3
CHCl3 ext.:
papaverine
narcotine
thebaine
laudanosine
unknown A
Aqueous
added
CHCl2COOH
extd. with CHCl3
CHCl3 ext.:
narceine
neopine
laudanine
laudanidine
unknowns B, C. D
Aqueous:
morphine
codeine, etc.
Evapd. to dryness
Residue dissolved in
0.1 N NaOH
extd., with CHCl3
CHCl3 ext.:
laudanine
laudanidine
unknowns C, D
Aqueous
+ NaHCO3
extd. with ether
ether phase:
unknown B
= Rekwline

EXAMPLES

Example 1. Mutation

Seeds of *Papaver somniferum* were obtained of about uniform size, dried to about 8% LOD (loss on drying) and placed in a mesh polyethylene bag at a weight of about 5 grams or about 12,500 per bag. The seeds were pre-soaked in beakers of distilled water containing a phosphate buffer at room temperature for about 36 hours. The seeds were given a further presoaking in cold 0.3% v/v (~0.028 M) ethyl methanesulphonate (EMS). Immediately after pre-soak, the seed bags were immersed in a mutagen bath containing 0.3% v/v (~0.028 M) ethyl methanesulphonate (EMS) at 20°C for 6 hours. Immediately following the mutagen bath, the seed bags were post-washed in running tap water. Following post-wash, the seeds were kept moist and planted within one hour.

Example 2. Propagation

The seeds were planted in outdoor plots and grown to maturity. The planting technique employed was in all respects normal for poppy trial work, and similar to commercial poppy growing. The seeds were sown using a "cone seeder" or trial plot drill. Seed depth was about 1 cm. Fertiliser containing N, P and K was used. The plots were irrigated immediately after sowing. The poppy flowers were self-pollinated and the majority of the flowers were covered with paper bags of bleached white "kraft" paper to prevent cross pollination. Seeds were harvested from those M1 generation plants which grew vigorously and appeared healthy. A second, M2, generation was grown from the harvested seeds. These seeds were planted in trays containing 200 plants. When the M2

plants were between the 10 leaf stage and the "running-up" stage, about 6 inches high, they were screened for alkaloid content using a rapid HPLC technique.

Example 3. Screening

The screening process was basically a three step process. In the first step, a leaf was cut from an M2 plant and about 0.5 μL of latex was collected at the wound. The latex was diluted in a microcentrifuge tube with 250 μL of buffer. The buffer contained 0.2 M ammonium phosphate, 20% ethanol, and had a pH of 4.5. The microcentrifuge tube was briefly held to a vortex shaker to ensure mixing. In the second step, the buffered solution was centrifuged to substantially eliminate suspended solids and about 200 μL was decanted into a 40 mm x 8 mm autoanalyser tube. Additional buffer, 250 μL, was added to-each auto analyser tube so that the sampling needle of the autoanalyser could reach the solution. In the third step, the autoanalyser tubes were loaded into a 96 place carousel inserted into the auto injector module of a Waters HPLC system. The HPLC mobile phase was aqueous methanol (approximately 30%) containing ammonium acetate buffer (0.08-0.12 M), pH 4-5. The flow rate of the mobile phase was 0.8-1.5 mL/minute. A Whatman Partisphere SCX column (4.6 x 125 mm) was used at a temperature of 40°C. A Waters 440 UV detector was used to detect the peaks at 254 nm. The data was interpreted and collated on a Waters Millennium Data Station. The system was used to analyse for alkaloids.

Two plants E40 and E41, were screened and found to be morphine and thebaine free and contained a peak later identified as (S)-reticuline. The two plants were combined and about 0.15 g of straw was harvested. The (S)-reticuline content was 3.3%, with 0.25% codeine and 0.007% thebaine. The reticuline was identified by circular dichroism as (S)-reticuline.

A descendant generation was grown in the field. The plants grew well, but two distinct types of plant were observed at the green capsule stage, those having white latex (E40/41W) and those having red latex (E40/41 R). From the variety with white latex was harvested 50.7 g of straw containing 3.88% (S)-reticuline and 0.78% codeine (or codeine-like alkaloids). The variety with red latex was observed to have 2.51% (S)-reticuline and zero codeine.

Example 4. Extraction

An acidic extract (pH 1.5) of opium is or concentrate of poppy straw is obtained in the usual manner. This acidic fraction is extracted with chloroform, which removes a number of alkaloids including papaverine, narcotine, thebaine and laudanosine. The acidic aqueous phase is then treated with dichloroacetic acid and further extracted with chloroform. Morphine and codeine remain in the aqueous phase but a number of alkaloids, including (S)-reticuline, partition into the organic phase. The organic phase is subsequently evaporated to dryness and the residue dissolved in 0.1 M NaOH. Laudanine and laudanidine partition into the chloroform layer. The aqueous layer is treated with sodium bicarbonate and the resultant aqueous layer extracted with ether. The ether layer is found to contain (S)-reticuline.

Example 5. Analysis

A HPLC trace of an E40R/41R extract is shown in Fig 1. The extract alone is the bottom trace, while the top trace is an solution containing extract and standards. (S)-reticuline is shown as having a retention time of about 16 minutes.

Example 6. Calculation

Phenanthrene alkaloids are those incorporating the phenanthrene ring system into their structure. Morphine is an example of such a phenanthrene type alkaloid.

Reticuline however does not include this in it's structure but has the "benzyl-isoquinoline" structure as its major structural element.

In the threshed straw of commercial poppies grown in Australia, (S)-reticuline constitutes no more than 0.04%, and the sum of all the phenanthrene alkaloids (morphine, codeine, thebaine and oripavine) is of the order of 1.2-2.7%, depending on the variety grown and factors such as crop nutrition and rainfall received.

Thus, 0.04/2.0 X 100 = 2%

In the reticuline poppies, the concentration of (S)-reticuline in the threshed poppy straw is about 2.5%, whereas the concentration of the sum of the phenanthrene alkaloids is at best 1.0%.

Thus, the percentage ratio is 2.5/1.0 X 100 = 250%

Dated this 14th Day of January, 1998

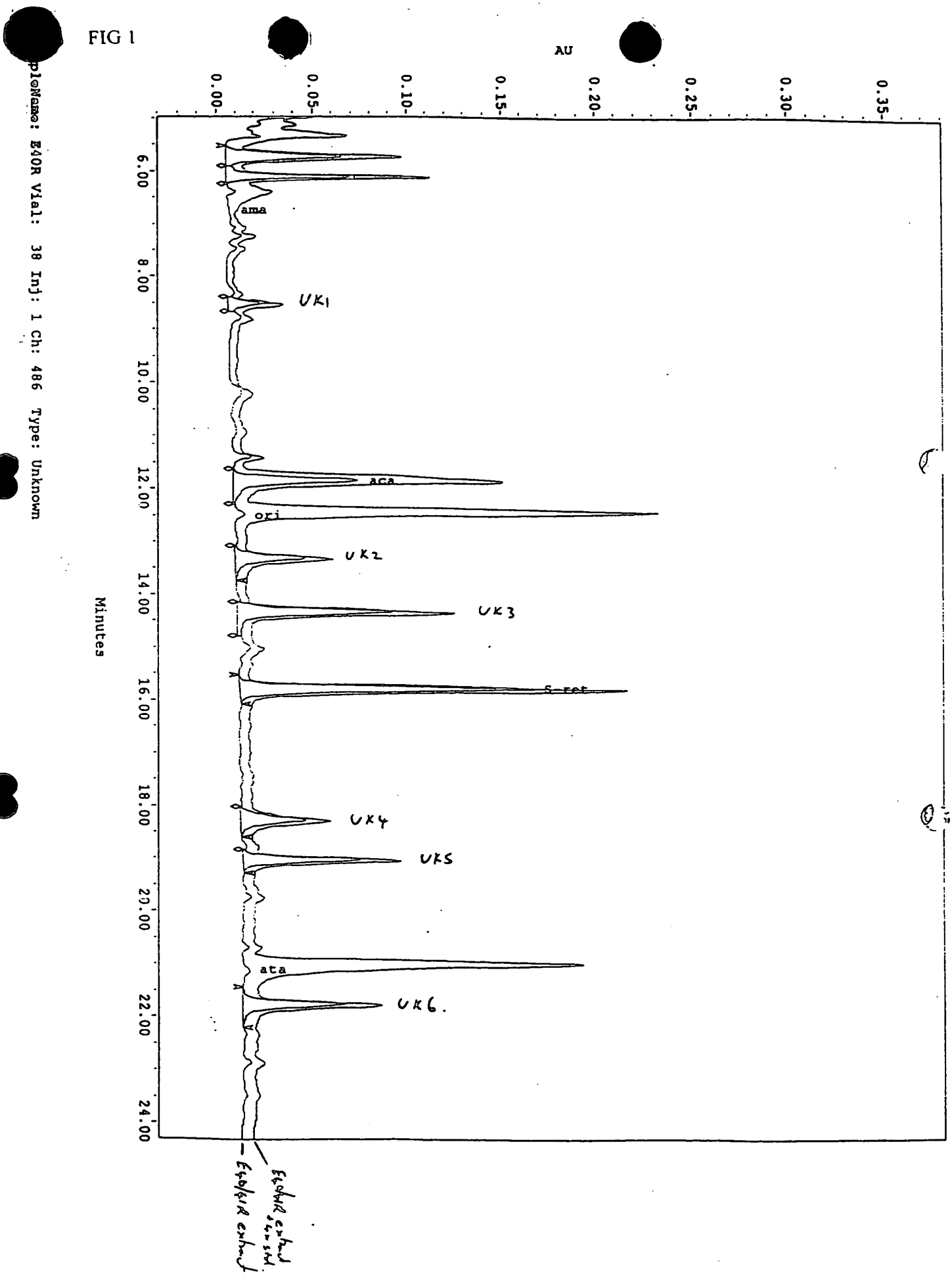
TASMANIAN ALKALOIDS PTY. LTD.

Attorney: IAN T. ERNST

Fellow Institute of Patent Attorneys of Australia

of SHELSTON WATERS

FIG 1


SampleName: E40R Vial: 38 Inj: 1 Ch: 486 Type: Unknown
AU
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
Minutes
ama
UK1
aca
ori
UK2
UK3
S-ret
UK4
UK5
ata
UK6.
E40R extract
E40/61R extract